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Methods for etiologic and early marker investigations in the PLCO trial

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Abstract

With the rapid development of biomarkers and new technologies, large-scale biologically-based cohort studies present expanding opportunities for population-based research on disease etiology and early detection markers. The prostate, lung, colorectal and ovarian cancer (PLCO) screening trial is a large randomized trial designed to determine if screening for these cancers leads to mortality reduction for these diseases.

Within the Trial, the PLCO etiology and early marker study (EEMS) identifies risk factors for cancer and other diseases and evaluates biologic markers for the early detection of disease. EEMS includes 155,000 volunteers who provide basic risk factor information. Serial blood samples are collected at each of six screening rounds (including one collection for cryopreserved whole blood) from screening arm participants (77,000 subjects) and buccal cells are collected from those in the control arm of the trial.

Etiologic studies consider environmental (e.g., diet), biochemical, and genetic factors. Early detection studies focus on blood-based biologic markers of early disease. Clinical epidemiology is also an important component of the PLCO trial.
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1. Introduction

The prostate, lung, colorectal and ovarian cancer (PLCO) screening trial is a large randomized trial to determine if screening for these cancers results in

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Table 1
Material collections in PLCO etiology and early marker study

	Screening arm	Projected subjects	Completion year
General questionnaire	Both	155000	2001
Diet questionnaire 1	Screen	72000	2001
Diet questionnaire 2	Both	155000	2004
Year 0 blood	Screen	72000	2001
Year 1 blood	Screen	71000	2001
Year 2 blood	Screen	69000	2003
Year 3 blood	Screen	68000	2004
Year 4 blood	Screen	68000	2005
Year 5 blood	Screen	67000	2006
Buccal cells	Control	77000	2004

reductions in cause-specific mortality for these diseases [1]. The Trial is carried out by the NCI under contract with investigators at ten clinical centers in the United States, including approximately 155,000 volunteers, recruited from 1992 through 2001, ages 55–74, equally distributed to the screening and control arms of the trial. To support etiologic and early detection studies of cancer and other diseases, the PLCO etiology and early marker study (EEMS) collects baseline demographic and risk factor information on all participants, food frequency questionnaires (FFQ) on all participants, fractionated blood specimens at each of six screening rounds from screened participants, and buccal cell samples from control participants (Table 1). All-cancer incidence and all-causes mortality data are collected for participants, based on annual follow-up surveys and other sources [2].

The PLCO EEMS is directed towards meeting the National Cancer Institute's strategic priorities in molecular epidemiology and early cancer detection. The NCI maintains follow-up of cohort members, initiates additional risk factor and biologic materials collection, including collection of pathologic material, and maintains a PLCO EEMS research database and sample inventory. In conjunction with research activities, resource enrichment includes validation of biochemical and cytogenetic markers, tissue arrays for pathologic samples (in planning), nutritional modules for FFQs, and assays for genetic analysis (http://snp500cancer.nci.nih.gov).

With the rapid development of biomarkers and new technologies, large-scale biologically-based cohort studies, such as PLCO EEMS, are presented with growing opportunities for productive research on disease etiology and early detection markers. Here we describe key facets of this complex epidemiologic endeavor.

2. Strategic planning

A strategic plan for etiologic and early marker investigations in PLCO EEMS is under development. It will guide research priorities and their implementation, involving NCI scientists, PLCO screening center investigators, and other extramural scientists, with an emphasis on collaboration among multiple groups. Strategic planning involves prioritization and coordination, which is crucial to maximize sample utility and to minimize sample deterioration (through uncoordinated sub-aliquotting). Study timing is also crucial; to assure that investigations are launched only when adequate sample sizes are achieved.

To accommodate these and other principles in meeting the strategic planning goals for etiologic studies, NCI will coordinate investigations of multiple (and inter-related) environmental (including lifestyle), genetic, and biochemical risk factors for cancer and related diseases. These investigations will use common study designs, taking full advantage of the extensive questionnaire and biologic sample base. As a key feature of strategic planning, studies in collaboration with investigators leading other large cohorts are planned to study complex etiologic relationships and rare disease outcomes.

Because the PLCO trial stores sequential prediagnostic blood samples, it is a unique resource for penultimate evaluations of promising candidates (i.e., prior to prospective studies and randomized trials) [3]. Because of the limited quantity of materials, high prior probabilities are required for their use. Evidence regarding sensitivity and specificity of the marker in clinical cancer series or cross-sectional studies is of value and, preferably, operating characteristics of the cancer marker will have been established in subjects with localized versus advanced cancer and in subjects with organ-specific benign conditions (organ-related false positives). Strategic planning includes opportunities for exceptional investigations not foreseen in the planning, if they do not undermine the NCI strategic goals by excessive sample depletion. Selected materials will also be dedicated to new high-priority technology applications.

3. Genetics and cancer etiology

A major purpose of EEMS PLCO is to relate genetic heterogeneity to disease risk, particularly for cancer. The approach to genetic epidemiology is evolving rapidly. Our focus has largely been on candidate genes, with particular attention to molecular pathways, e.g. inflammation, sex hormones, growth factors, endogenous activation and deactivation of carcinogens, insulin resistance, and several nutrition-related pathways. The rational for the candidate gene approach is that polymorphisms in candidate genes affect the function of the encoded proteins, e.g. enzyme activity or receptor binding and thereby alter disease risk. For instance, genetic polymorphisms in sex hormone-metabolizing enzymes potentially affect risk of hormone-related cancers, or genetic polymorphisms in folate-metabolizing enzymes impact colon cancer risk.

Selection of polymorphisms, mainly single nucleotide polymorphisms (SNPs), relies on publicly available data as well as focused re-sequencing of specific genes in a sufficient number of ethnicity-specific subjects to characterize population variation of relatively frequent gene variants (e.g., >5%). SNPs for genotyping are selected based on functional considerations or as markers to capture common variation within a gene and its regulatory regions [4,5]. Important resources for gene variation identification are listed in Table 2.

With an increasing number of studies investigating associations between various candidate genes and cancer risk, it is becoming evident that many individual genetic variants result only in small relative risks, as primary factors or in interaction with environmental exposures (gene–environment interactions). To investigate small relative risk and gene–environment interactions, large samples sizes are needed. For example, the PLCO study is part of the breast and prostate cancer and hormone-related gene variants cohort consortium, which combines six large cohort studies to investigate gene–environment interactions in more than 6000 breast cancer cases and almost 9000 prostate cancer cases. Furthermore, as understanding of the human genome increases and assay costs decrease, targeted evaluation of inherited variation in rapidly evolving repeat sequences [6] and transcriptional regulation [7] pathways, as well as non-targeted genome scans are becoming feasible.

4. Serum-biomarkers and cancer etiology

Prospectively collected blood specimens of PLCO EEMS provide the opportunity to study various biomarkers in relation to cancer risk, for example, to assess dietary intake for nutrients which cannot be reliably assessed by questionnaire (e.g., vitamin D and selenium) or to better specify observed diet-cancer associations. Blood specimens are also important to assess endogenous biological markers (e.g., cytokines, C-reactive protein, hormones, growth factors, insulin, glucose, and adducts). The focus in PLCO is on new biomarker-cancer associations and verifying previously observed associations. Given the prospective design and study participant numbers, PLCO EEMS can contribute substantially to the overall epidemiological evidence.

As samples collected prospectively in epidemiologic studies are extremely valuable, it is important to ensure high quality throughout the analysis of bio-

Table 2
Publicly available human genetic variation resources

Database name	URL	Description
NIEHS environmental genome project	http://egp.gs.washington.edu/welcome.html	Resequencing data of candidate genes particularly environmental response genes, e.g. DNA repair and cell cycle pathways
UW-FHCRC variation discovery resource	http://pga.gs.washington.edu/	Resequencing data of candidate genes, particularly genes related to inflammation
dbSNP	http://www.ncbi.nlm.nih.gov/SNP/index.html	Central repository for SNPs, short deletion and insertion polymorphisms
HapMap	http://www.hapmap.org/index.html.en	Resequencing data of all chromosomes
SNP500	http://snp500cancer.nci.nih.gov/home_1.cfm	Sequence verification of SNPs known and newly discovered SNPs

logical markers, including monitoring of assay quality prior to the start of, early into, and throughout the analysis of study samples. We routinely test markers in assay reproducibility studies (laboratory-blinded assay of repeat samples), followed by assay "run-in" of the first analytic batch with added quality control (QC) evaluation, and systematic blinded QC inclusion in the full analytic batches. As assay results are reported, QC evaluation is an ongoing process, to allow for rapid intervention if reproducibility falls from accepted standards.

Given that many PLCO studies include one- to two-thousand samples and are often using relatively low-throughput assays, sample analysis can take many months to complete. Keeping all variables constant (e.g. assay materials, and personnel) throughout the analysis provides an additional challenge for the analytical laboratory and emphasizes the importance of monitoring quality closely, with the by-product of documentation of assay reliability of analytical methods [8].

A further quality control issue is assuring that the least amount of sample is used which will give an accurate result. As part of the pilot phase, it is important to get detailed information about the assay, e.g. the actual amount of sample used in a single assay, dead space requirements, failure rate of the assay, and to discuss with the analytical laboratory potential sources to reduce failure rates (e.g. no automated runs during the night). If the failure rate can be kept low, it is sufficient to send sample for only one run. In addition, when we aliquot the parent vial into multiple daughter vials for various assays we set an aliquot aside to be used if an assay fails for some of the PLCO subjects. This way we can send material for only one run, even for assays with relatively higher failure rates.

5. Viable cells in the PLCO trial

Preservation of viable cells in large studies presents challenges of maximizing multiple endpoint versatility with budgetary constraints and responsible resource conservation. The PLCO screening trial provides one of the rare opportunities to interrogate viable cells collected from persons prior to a cancer diagnosis and disease-free controls.

To address concerns about a single central laboratory processing large numbers of biospecimens from geographically dispersed collection centers and to assess long-term storage of whole blood step-cryopreserved with DMSO, 169 samples were evaluated by several methods after storage in the vapor phase of liquid nitrogen for variable lengths of time [9]. Lymphocyte viability, T-cells as a percentage of total lymphocytes, T-cell/B-cell ratio, and lymphocyte stimulation were evaluated at several time-points up to 2.5 years of cryopreservation, showing only minor effects of long-term storage. Of 60 samples that were stored for up to 20 months, 92.5% were successfully immortalized into normal lymphoblastoid cell lines by EBV transformation.

The prospect of applying functional susceptibility assays or tests for early detection, using the cryopreserved whole blood resource, is clearly one of the most exciting aspects of the PLCO Screening Trial. Additionally, creating an inexhaustible source of viable cells by EBV transformation from well characterized cancer case and control individuals can only add to the study's resource value.

6. Viable cells and cancer etiology

The main rationale for using cytogenetic assays for biological monitoring is that genetic damage in a non-target tissue, such as peripheral blood lymphocytes, is thought to reflect similar events in target tissue and can therefore serve as early indicators of DNA damage, with chromosome aberrations (CA) serving as integrative biomarkers that reflect exposure to chromosome-damaging carcinogens as well as host factors, such as procarcinogen activation and detoxification, and DNA repair. Several small studies from a wide range of retrospective resources have explored the role of non-clonal cytogenetic markers as predictors of future human cancer [10].

Technologies such as fluorescent in situ hybridization (FISH) enable the evaluation of specific aberrations in a large number of metaphase cells [11,12] and samples collected following the PLCO trial cryopreservation protocol are suitable for FISH analysis. Several investigators worldwide have used functional tests (e.g. G_2 or mutagen sensitivity, host-cell reactivation, and Comet assays) in case-control studies

to evaluate human cancer risk associated with DNA damage and DNA damage repair capacity (DRC), suggesting that elevated DNA damage induced by in vitro mutagen exposure (or reduced DRC) was associated with increased cancer risk at several sites (e.g. head and neck, lung, brain, breast, bladder, melanoma, and non-melanoma skin cancer) [13–16]. The predictive power of these assays is, however, uncertain because of the potential for reverse-causation bias, as tests were performed on biologic specimens collected after cancer diagnosis and may be measuring the consequence rather than the underlying causes of cancer. Prospective evaluations with viable cells in PLCO could address this potential bias.

Other potential biomarkers include candidate protein levels (Ku70, Ku86, DNAPK, and the telomerases) and several variations of FISH developed to detect damage-specific events, such as mBAND for high-LET radiation [17] and CO-FISH for telomere–telomere and telomere–double strand break fusion events [18]. Presently several of these assays are laborious and expensive, but with continued technologic improvement may be amenable for application in human epidemiologic studies.

7. Blood-based early detection studies

Early detection of cancer involves the identification of pre-malignant and malignant lesions at a sufficiently early stage in development, such that clinical intervention leads to a reduction in cancer-related morbidity and mortality. Biomarkers for the early detection of cancer may include any cellular, biochemical, or genetic abnormality that aids this process. Early detection by differential serum proteomic profile analysis shows great promise [19–21], however several aspects of the analysis remain challenging. First, careful data pre-processing, including calibration, standardization and normalization of spectra is a crucial step in analyzing proteomic data [22]. Second, the large number, of features for each profile combined with small sample sizes (typically less than 100) may lead to over-fitting of data and resulting non-reproducible results [23], and proteome profiles are highly complex, requiring novel statistical algorithms, including machine-learning techniques [24,25].

New high-throughput approaches are being developed for the assessment of multiple protein markers in biologic samples [26,27]. As the technology for multiple identified marker assessment improves, the proteomic and protein-specific approaches will converge, providing broad-based profiles associated with well-characterized early disease markers, although data complexity issues will remain a significant challenge.

The PLCO trial offers unique opportunities for the study of the natural history of blood-based early markers of cancer, particularly due to the serial collection of blood specimens annually for 6 years. The operating characteristics of promising early markers of cancer will be assessed, with respect to cancer and pre-malignant disease (particularly pertinent for the PLCO tumors) using nested case-control studies in the PLCO trial. The nested case-control design uses an efficient sampling of subjects to determine true positive and false positive rates, at varying levels of the biomarker. Because of the serial collections, it will be possible to assess changes in these operating characteristics, with respect to time prior to disease diagnosis; markers that have a longer lead time prior to disease diagnosis should be better candidates for early detection. Studies of marker time-dependent operating characteristics will be crucial for the subsequent development of randomized screening trial protocols (e.g., marker positive cut-points and screening cycle times) for evaluation of marker-related morbidity and mortality reduction [3].

There is limited information on sources of potential bias and the process of developing and testing predictive algorithms in well characterized prospective control samples. Several key issues are pertinent, including storage artifacts, laboratory handling (e.g., number of freeze–thaw cycles and the effect of hemolysis), demographic confounders (e.g., age, dietary factors), and markers that are predictive of disease status only due to non-specific factors (e.g., nutritional status, immunodeficiency, and inflammation). Because serum samples from PLCO are not vulnerable to many of the biases that arise in specimen from clinical sources, the sample resource provides unique opportunities for assay development, reliability testing, and evaluation of assay operating characteristics in distinguishing (pre-diagnostic) cases from controls.

8. Clinical studies

The primary goal of the PLCO as a clinical trial is to assess whether screening for the PLCO cancers reduces mortality from those cancers. However, the PLCO study also provides an excellent opportunity to perform clinical research on other topics related to screening for and diagnostic follow-up of the PLCO cancers.

One area of research involves characteristics of the screening tests per se. For example, Gelmann et al. [28] characterized total PSA and free PSA levels by age and race in PLCO, finding that older men had higher levels of total PSA and higher levels of free PSA than younger men, but relatively similar percent free PSA; a similar pattern was found for blacks versus whites. Crawford et al. [29] found very low rates (1.4%) of conversion to PSA above 4 ng/ml within 5 years in men with baseline PSA below 1.0 ng/ml and Pinsky et al. [30] described factors that influence screen positive men to receives a prostate biopsy. Research has also been carried out on the variability among examiners in flexible sigmoidoscopy performance and in how nurse practitioners versus gastroenterologists compare in sigmoidoscopy performance [31,32].

Another area of focus is on pre-cancerous lesions, especially colorectal adenomas. Pinsky et al. [33] showed that subjects with distal hyperplastic polyps and distal non-advanced adenomas had similar rates of advanced proximal neoplasia, but that subjects with advanced distal adenomas had about a 2.5-fold higher rate of advanced proximal neoplasia. Schoen at al. [34] showed that 0.8% of subjects examined by sigmoidscopy at the 3 years follow-up after a negative baseline screen had a distal advanced adenoma or cancer. Another mechanism for clinical research in PLCO is through ancillary studies requiring additional materials collection, such as ongoing work on CT colonography, [35,36] aberrant crypt foci, and surveillance colonoscopy in the PLCO population.

9. Summary

The PLCO EEMS carries out etiologic and early marker investigations in a nation-wide cancer early detection trial. The PLCO EEMS is directed towards meeting the National Cancer Institute's strategic priorities in molecular epidemiology and early cancer detection. The next decade will see a great expansion of integrative studies, employing new technologies in epidemiologic cohorts to increase our understanding of etiologic factors and means for disease prevention. The PLCO EEMS, through its unique resources, is positioned for these multi-disciplinary investigations.

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